



# Cautionary Notes on the Use of Arabinose- and Rhamnose-Inducible Expression Vectors in *Pseudomonas aeruginosa*

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**ABSTRACT** The *Pseudomonas aeruginosa* virulence factor regulator (Vfr) is a cyclic AMP (cAMP)-responsive transcription factor homologous to the *Escherichia coli* cAMP receptor protein (CRP). Unlike CRP, which plays a central role in *E. coli* energy metabolism and catabolite repression, Vfr is primarily involved in the control of *P. aeruginosa* virulence factor expression. Expression of the Vfr regulon is controlled at the level of *vfr* transcription, Vfr translation, cAMP synthesis, and cAMP degradation. While investigating mechanisms that regulate Vfr translation, we placed *vfr* transcription under the control of the *rhaBp* rhamnose-inducible promoter system (designated P<sub>Rha</sub>) and found that P<sub>Rha</sub> promoter activity was highly dependent upon *vfr*. Vfr dependence was also observed for the *araBp* arabinose-inducible promoter (designated P<sub>BAD</sub>). The observation of Vfr dependence was not entirely unexpected. Both promoters are derived from *E. coli*, where maximal promoter activity is dependent upon CRP. Like CRP, we found that Vfr directly binds to promoter probes derived from the P<sub>Rha</sub> and P<sub>BAD</sub> promoters *in vitro*. Because Vfr-cAMP activity is highly integrated into numerous global regulatory systems, including c-di-GMP signaling, the Gac/Rsm system, MucA/AlgU/AlgZR signaling, and Hfq/sRNAs, the potential exists for significant variability in P<sub>Rha</sub> and P<sub>BAD</sub> promoter activity in a variety of genetic backgrounds, and use of these promoter systems in *P. aeruginosa* should be employed with caution.

**IMPORTANCE** Heterologous gene expression and complementation constitute a valuable and widely utilized tool in bacterial genetics. The arabinose-inducible P<sub>araBAD</sub> (P<sub>BAD</sub>) and rhamnose-inducible P<sub>rhaBAD</sub> (P<sub>Rha</sub>) promoter systems are commonly used in *P. aeruginosa* genetics and prized for the tight control and dynamic expression ranges that can be achieved. In this study, we demonstrate that the activity of both promoters is dependent upon the cAMP-dependent transcription factor Vfr. While this poses an obvious problem for use in a *vfr* mutant background, the issue is more pervasive, considering that *vfr* transcription/synthesis and cAMP homeostasis are highly integrated into the cellular physiology of the organism and influenced by numerous global regulatory systems. Fortunately, the synthetic P<sub>Tac</sub> promoter is not subject to Vfr regulatory control.

**KEYWORDS** *Pseudomonas aeruginosa*, Vfr, cyclic AMP, arabinose, rhamnose

Bacteriologists study gene expression and function using genetics tools, including transcriptional and translational reporters, genetic deletions, engineered transposons, and complementation/overexpression vectors. The gold standard for establishing gene function is to generate an in-frame deletion mutant, assign a phenotype, and then complement the phenotype by exogenous expression of the deleted gene. A companion approach is to overexpress the gene of interest and screen for phenotypes. Complementation/overexpression vectors make use of heterologous promoters to

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drive expression of the cloned gene. The most versatile systems utilize tunable promoters with rheostat-like control, such as the arabinose-inducible *araBp* (designated  $P_{BAD}$ ) and rhamnose-inducible *rhaBp* (designated  $P_{Rha}$ ) promoters from *Escherichia coli*. Both promoters are controlled by a transcription factor (AraC and RhaS, respectively) that activates expression of genes required for the utilization of the sugars arabinose and rhamnose (1, 2). The same sugars serve as the inducing ligands for AraC- and RhaS-dependent activation. Ligand binding triggers conformational changes in AraC and RhaS that alter or enable their DNA-binding activity (3, 4). Expression vectors that incorporate these promoters allow variable expression of cloned genes in a manner that is directly proportional to the concentration of sugar added to the growth medium.

*E. coli* preferentially utilizes glucose as a carbon source. When it is growing in the presence of glucose, the genes required to transport and catabolize other sugars (such as arabinose or rhamnose) are repressed, even when the alternative sugars are present, through a process referred to as carbon catabolite repression (5). Carbon catabolite repression is controlled by the second messenger cyclic AMP (cAMP) and CRP (cyclic AMP receptor protein). Whereas cAMP levels are low when *E. coli* is growing on glucose, cAMP levels increase when glucose is depleted (6). cAMP-bound CRP binds to specific sites within or near target promoters and recruits RNA polymerase (RNAP) through interactions with the  $\alpha$  subunit of RNAP and/or the sigma factor (7–13). Transcription of the *E. coli* arabinose or rhamnose utilization genes is coupled to carbon catabolite repression by CRP (2, 14–19). CRP-cAMP binds near divergent promoters that control *araC* and the *araBAD* operon and promotes expression from both promoters (19). Similarly, the divergent *rhaRS* and *rhaBAD* operons are activated by RhaR and RhaS, respectively, and each promoter region has a CRP-cAMP binding site (2, 20, 21). Maximal expression of arabinose or rhamnose utilization genes, therefore, requires both the specific inducing sugar and elevated cAMP levels (i.e., low glucose levels).

*Pseudomonas aeruginosa* encodes a CRP family protein called Vfr (virulence factor regulator). Rather than participating in carbon catabolite repression (22), Vfr regulates many virulence-related genes, including exotoxin A production (23, 24), the *las* quorum sensing system (25), type IV pilus biogenesis (26), and the type III secretion system (27, 28). Vfr and CRP both interact with DNA, RNAP, and cAMP and share 61% identity and 91% similarity at the amino acid level (23). Like CRP, Vfr usually requires cAMP for DNA-binding activity, with the noted exception of cAMP-independent binding to the *lasR* promoter region (23). Whereas exogenous *vfr* expression complements an *E. coli* *crp* mutant, *crp* expression does not fully complement a *vfr* deletion mutant (23).

Genetics tools that make use of the  $P_{Rha}$  and  $P_{BAD}$  promoter systems are commonly used to study *P. aeruginosa*. While investigating translational control of Vfr, we constructed  $P_{Rha}$ -*lacZ* and  $P_{BAD}$ -*lacZ* transcriptional reporters and found that transcription from both promoters is strongly dependent upon *vfr*. Consistent with our genetic data, Vfr binds to  $P_{Rha}$  and  $P_{BAD}$  promoter regions with high affinity and specificity *in vitro*. Given the reliance of both promoters on CRP in *E. coli* and the high similarity between CRP and Vfr, our findings are not entirely unsurprising. Nevertheless, our findings demonstrate that utilization of the  $P_{Rha}$  and  $P_{BAD}$  promoter systems in *P. aeruginosa* is fraught with potential pitfalls, given that Vfr-cAMP activity is highly integrated into numerous global regulatory systems, including c-di-GMP signaling, the Gac/Rsm system, MucA/AlgU/AlgZR signaling, and Hfq/sRNAs (29–32). The *tac* promoter ( $P_{Tac}$ ) is a synthetic inducible system that was constructed by combining the *trp* and *lacUV5* promoters (33). While the *lac* operon requires CRP for full activation, *lacUV5* is no longer under CRP control (34). We find that  $P_{Tac}$  promoter activity is independent of Vfr and a suitable promoter system for regulated control of gene expression in *P. aeruginosa*.

## RESULTS

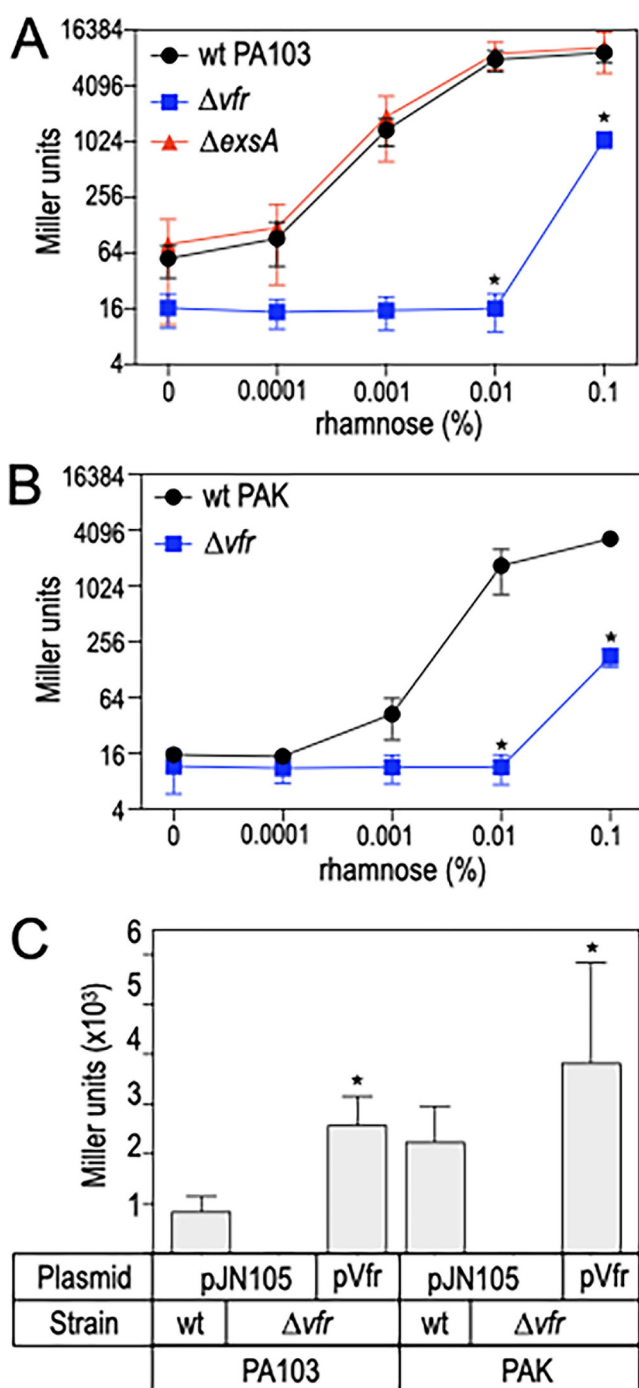
**$P_{Rha}$  and  $P_{BAD}$  promoter activity are dependent upon *vfr*.** Our studies on mechanisms of gene control in *P. aeruginosa* make use of the commonly employed  $P_{Rha}$ - and

$P_{BAD}$ -regulated expression systems. During the course of our studies, we began to suspect that  $P_{Rha}$  promoter activity was Vfr dependent. To examine the effect of *vfr* on  $P_{Rha}$  promoter activity, we constructed a  $P_{Rha}$ -*lacZ* transcriptional reporter and integrated the reporter into the Tn7 site of wild-type (wt) *P. aeruginosa* strain PA103 and a *vfr* deletion mutant ( $\Delta vfr$  strain). In wt PA103, the  $P_{Rha}$ -*lacZ* reporter demonstrated a dose-dependent increase in activity with the inducer rhamnose (Fig. 1A). In contrast, reporter activity was detected in the  $\Delta vfr$  strain only at the highest concentration of rhamnose (0.1%) tested, and even then, activity was more than 8-fold lower than observed for the wt strain (Fig. 1A). As a control, we also introduced the  $P_{Rha}$ -*lacZ* reporter into an *exsA* deletion mutant ( $\Delta exsA$  strain), required for type III secretion gene expression. We expected to see no effect and found that reporter activity was similar to the wt strain (Fig. 1A). Complementation of the  $\Delta vfr$  strain with a plasmid-encoded copy of *vfr* driven from a heterologous promoter restored  $P_{Rha}$ -*lacZ* reporter activity, confirming that the defect resulted from Vfr insufficiency (Fig. 1C). To verify that Vfr dependence of the  $P_{Rha}$ -*lacZ* reporter was not restricted to strain PA103, we introduced the reporter into wt strain PAK and a  $\Delta vfr$  mutant. The expression pattern was similar to that of strain PA103, wherein wt PAK demonstrated dose-dependent expression of the reporter, and activity in the  $\Delta vfr$  strain was observed only with the highest concentration of rhamnose tested (Fig. 1B). Providing a copy of *vfr* in *trans* restored  $P_{Rha}$ -*lacZ* reporter activity in the PAK  $\Delta vfr$  mutant (Fig. 1C).

Since the  $P_{BAD}$  promoter is also controlled by CRP in *E. coli*, we constructed and integrated a  $P_{BAD}$ -*lacZ* transcriptional reporter into the Tn7 site of wt PA103 and the  $\Delta vfr$  and  $\Delta exsA$  mutants. In the wt and  $\Delta exsA$  backgrounds  $P_{BAD}$ -*lacZ* reporter activity demonstrated a dose-dependent increase with the inducer arabinose (Fig. 2A). Whereas the  $\Delta vfr$  strain was completely devoid of reporter activity, the deficiency was rescued when *vfr* was provided in *trans* (Fig. 2B).

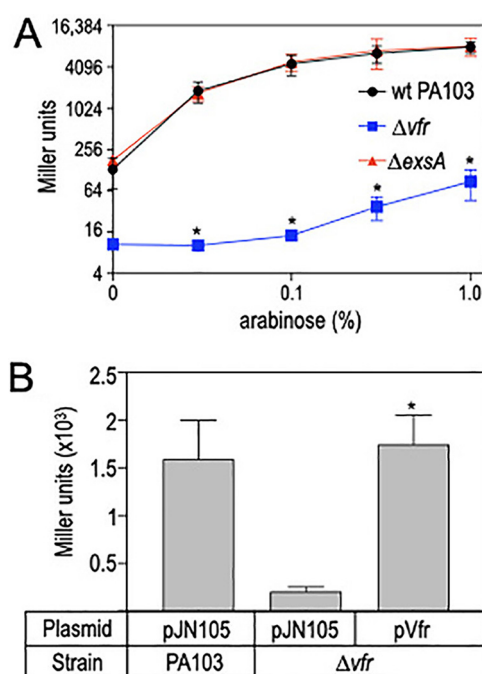
The  $P_{Rha}$ -*lacZ* and  $P_{BAD}$ -*lacZ* reporters described above are integrated into the chromosome in single copy at the Tn7 site. Many expression vectors are multicopy. To determine whether Vfr dependence of the  $P_{BAD}$  promoter is a peculiarity of being integrated at the Tn7 site and whether it is also observed in a plasmid-based system, we tested gene expression from the pJN105 vector. pJN105 is moderate-copy-number vector that utilizes the  $P_{BAD}$  promoter to control expression of cloned genes (35). We made use of the previously described ExsA-dependent reporter  $P_{exsD}$ -*lacZ* integrated at the CTX site (36) and expressed *exsA* in *trans* from the pJN105 vector (Fig. 3). Whereas  $P_{exsD}$ -*lacZ* reporter activity (Fig. 3A) and ExsA protein levels (Fig. 3B) demonstrated a dose-dependent response in an  $\Delta exsA$  mutant, reporter activity and ExsA levels were significantly reduced in a strain lacking both *exsA* and *vfr*. We conclude that Vfr dependence of the  $P_{BAD}$  promoter, and by inference the  $P_{Rha}$  promoter, is genuine, in agreement with the same requirements in *E. coli*, and does not reflect an artifact of being integrated in a single copy at the Tn7 site.

**Vfr directly binds to the  $P_{Rha}$  and  $P_{BAD}$  promoter regions.** A previous study in *Pseudomonas putida* also observed Vfr (CRP) dependence of the  $P_{Rha}$  promoter but concluded that the effect was indirect and resulted from a deficiency in rhamnose transport rather than a strict requirement for Vfr (37). This was demonstrated by expressing the rhamnose transporter (*rhaT*) in the *vfr* mutant and observing restoration of  $P_{Rha}$  promoter activity (37). To determine whether the same solution might work in *P. aeruginosa*, we cloned *rhaT* driven by the  $P_{Tac}$  promoter into the  $P_{Rha}$ -*lacZ* reporter and integrated the construct into the Tn7 site of wt PA103 and the  $\Delta vfr$  mutant. In the wt background, the  $P_{Rha}$ -*lacZ* reporter was significantly more responsive to rhamnose in the presence of *rhaT* (Fig. 4A, closed circles) than the construct lacking *rhaT* (open circles), demonstrating that *rhaT* is functional in *P. aeruginosa* and increases the intracellular rhamnose concentration. The  $P_{Rha}$ -*lacZ* reporter in the  $\Delta vfr$  mutant was similar in both the absence (Fig. 4A, open squares) and presence (closed squares) of *rhaT*. We conclude that while rhamnose transport is clearly limiting in the absence of *rhaT*, that does not fully account for Vfr (CRP) dependence of the  $P_{Rha}$  promoter.



**FIG 1**  $P_{Rha}$  promoter activity is Vfr dependent. (A and B) *P. aeruginosa* strains PA103 (A) and PAK (B) carrying a chromosomally integrated  $P_{Rha}$ -*lacZ* transcriptional reporter were cultured in LB with the indicated concentrations of rhamnose. When the culture  $A_{600}$  reached 1.0, the cells were harvested and assayed for  $\beta$ -galactosidase activity, reported in Miller units. \*,  $P < 0.05$  (ANOVA). (C) The indicated PA103 and PAK strains carrying the  $P_{Rha}$ -*lacZ* reporter, transformed with a vector control (pJN105) or a Vfr expression vector (pVfr), were cultured in LB with 0.005% rhamnose. Cells were assayed for  $\beta$ -galactosidase activity as described above. The reported data are the averages from at least three independent experiments. \*,  $P < 0.05$  (ANOVA).

The simplest explanation for Vfr dependence is direct binding of Vfr to the  $P_{Rha}$  and  $P_{BAD}$  promoter regions and facilitation of RNAP recruitment. We tested this hypothesis *in vitro* using an electrophoretic mobility shift assay (EMSA). Promoter probes (~200 bp) encompassing the  $P_{Rha}$  and  $P_{BAD}$  promoter regions and a nonspecific portion of *algD*

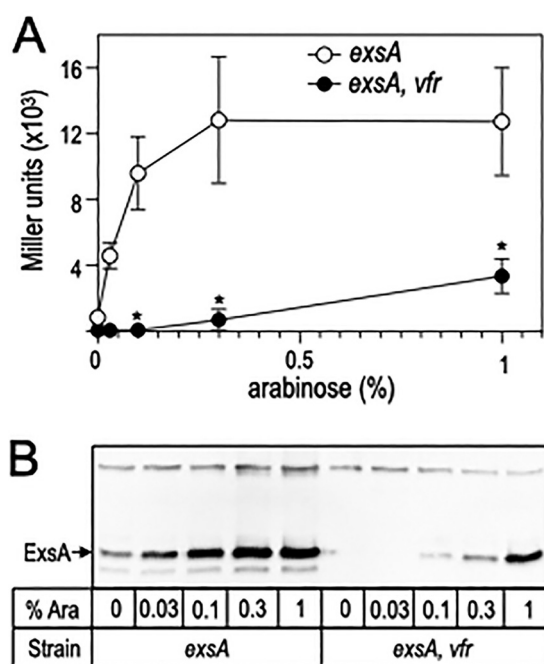


**FIG 2**  $P_{BAD}$  promoter activity is Vfr-dependent. (A) PA103 strains carrying a chromosomally integrated  $P_{BAD}$ -*lacZ* transcriptional reporter were cultured in LB with the indicated concentrations of arabinose. When the culture  $A_{600}$  reached 1.0, the cells were harvested and assayed for  $\beta$ -galactosidase activity, reported in Miller units. (B) PA103 or a  $\Delta vfr$  mutant carrying the  $P_{BAD}$ -*lacZ* reporter, transformed with a vector control (pJN105) or a Vfr expression vector (pVfr), was cultured in LB. The culture was back-diluted to a  $A_{600}$  of 0.8 in LB with 0.2% arabinose and incubated for an additional 45 min. The cells were then harvested and assayed for  $\beta$ -galactosidase activity. The reported data are the averages from at least three independent experiments. \*,  $P < 0.05$  (ANOVA).

lacking a Vfr binding site ( $\sim 180$  bp) were radiolabeled, incubated with Vfr, and analyzed by nondenaturing gel electrophoresis. Both the  $P_{Rha}$  (Fig. 4B) and  $P_{BAD}$  (Fig. 4C) promoter probes demonstrated retarded mobility when incubated with increasing concentrations of Vfr. Formation of the Vfr- $P_{Rha}$  and Vfr- $P_{BAD}$  promoter probes complexes was specific, as Vfr binding to the *algD* probe was not evident, even with the highest concentration of Vfr tested (200 nM) (Fig. 4C, lane C). Vfr- $P_{Rha}$  also formed a second lower-mobility complex at the higher concentrations of Vfr (Fig. 4B). This is consistent with the presence of multiple CRP binding sites in the  $P_{Rha}$  region (21).

**Example of a global regulator that impacts  $P_{Rha}$  promoter activity.** We recently demonstrated that Hfq and the small noncoding RNA 179 inhibit cAMP-Vfr activity (Fig. 5A) (32). In agreement with that finding,  $P_{Rha}$ -*lacZ* and  $P_{BAD}$ -*lacZ* reporter activities are elevated in an *hfq* deletion mutant relative to wt strain PA103 (Fig. 5B and C), and Hfq overexpression inhibits  $P_{Rha}$ -*lacZ* reporter activity (Fig. 5D).

**Stability of pUC18-mini-Tn7T-Gm-LacZ10-encoded  $\beta$ -galactosidase.** The  $P_{Rha}$ -*lacZ* and  $P_{BAD}$ -*lacZ* reporters were constructed in pUC18-mini-Tn7T-Gm-LacZ10 (obtained from Addgene). We observed that  $\beta$ -galactosidase expressed from this vector is labile relative to  $\beta$ -galactosidase expressed from mini-CTX-*lacZ*, another commonly used *P. aeruginosa* vector (Fig. 6). We also noted that a predicted BstZ171 restriction site located in the 3' end of *lacZ* does not digest pUC18-mini-Tn7T-Gm-LacZ10. Sequencing that plasmid revealed a base change that destroys the BstZ171 recognition site and results in a substitution of isoleucine for threonine at codon 928 of native *lacZ*. Replacing the 3' end of *lacZ* with the corresponding region from mini-CTX-*lacZ* restored  $\beta$ -galactosidase stability (Fig. 6). This is important to note, as  $\beta$ -galactosidase is generally assumed to be stable when cells are lysed in Z-buffer (as demonstrated for  $\beta$ -galactosidase expressed from mini-CTX-*lacZ*). For that reason, performing the enzymatic assay is not considered time critical. When using constructs derived from pUC18-mini-Tn7T-Gm-LacZ10, however, it would be critical to perform the enzymatic assay immediately upon cell harvest or to correct *lacZ*.



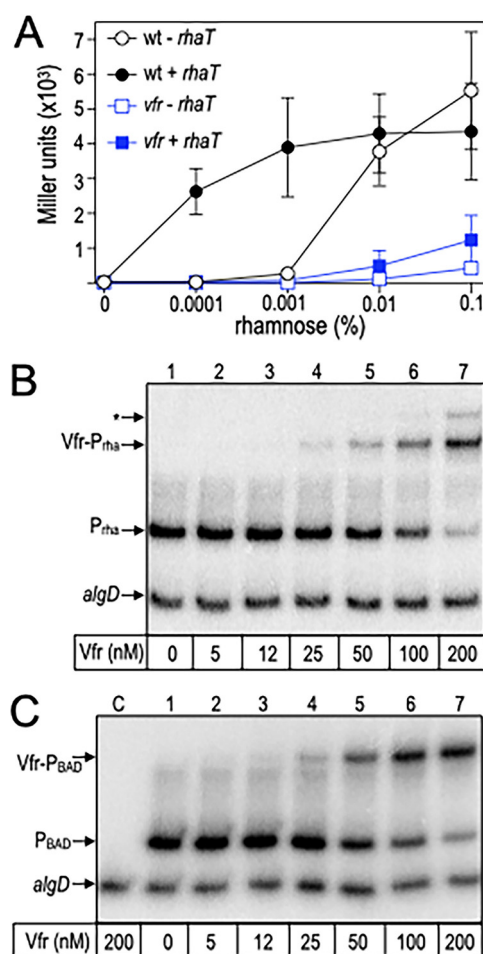
**FIG 3** Plasmid-based  $P_{BAD}$  promoter activity is Vfr dependent. PA103 *exsA* and *exsA vfr* strains carrying a chromosomally integrated  $P_{exsD}$ -*lacZ* transcriptional reporter were transformed with a plasmid encoding *exsA* under the transcriptional control of the  $P_{BAD}$  promoter. Cells were cultured in LB with the indicated concentrations of arabinose. When the culture  $A_{600}$  reached 1.0, the cells were harvested and assayed for  $\beta$ -galactosidase activity, reported in Miller units (A), and ExsA protein levels by immunoblotting using ExsA antiserum (B). The reported  $\beta$ -galactosidase activity data are the averages from at least three independent experiments. \*,  $P < 0.05$  (t test).

**$P_{Tac}$  promoter activity does not require Vfr.** One alternative to the  $P_{BAD}$  and  $P_{Rha}$  expression systems is the inducible  $P_{Tac}$  promoter.  $P_{Tac}$  is a synthetic promoter derived from the *E. coli* *trp* and *lacUV5* promoters that removes CRP control (33). Whereas  $P_{BAD}$  and  $P_{Rha}$  are reliant upon a sugar-dependent activator, the  $P_{Tac}$  promoter is controlled by the sugar-responsive repressor LacI (33). Binding of either allolactose or the gratuitous inducer IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) results in an allosteric change that releases LacI from an operator that overlaps the transcription start site. A  $P_{Tac}$ -*lacZ* reporter was generated and introduced at the Tn7 site of wt PA103 and the  $\Delta vfr$  and  $\Delta exsA$  mutants (Fig. 7). While there was a slight decrease in the  $\Delta vfr$  strain, this was not statistically significant. We conclude that the  $P_{Tac}$  system is appropriate to use in *P. aeruginosa* studies where *vfr* expression and activity may be altered.

## DISCUSSION

Regulated expression systems have many applications, including complementation experiments and the uncoupling of gene expression from native regulatory control. Our finding that the *E. coli*  $P_{Rha}$  and  $P_{BAD}$  promoters demonstrate strong Vfr dependence detracts from their utility for genetic studies in *P. aeruginosa*. For complementation experiments, where the objective is to verify that a phenotype is attributable to loss of a particular gene, this may not be a major issue, though the levels of gene expression achieved should be experimentally determined in each new genetic background. More problematic are experiments that require, and make the assumption of, equal/similar transcription levels in different genetic backgrounds. While that assumption is clearly erroneous in a  $\Delta vfr$  mutant, any mutant background or growth condition that impacts *vfr* transcription, Vfr synthesis, or cAMP homeostasis would suffer from the same limitation, and there is no shortage of genes that directly or indirectly impact the Vfr-cAMP system.

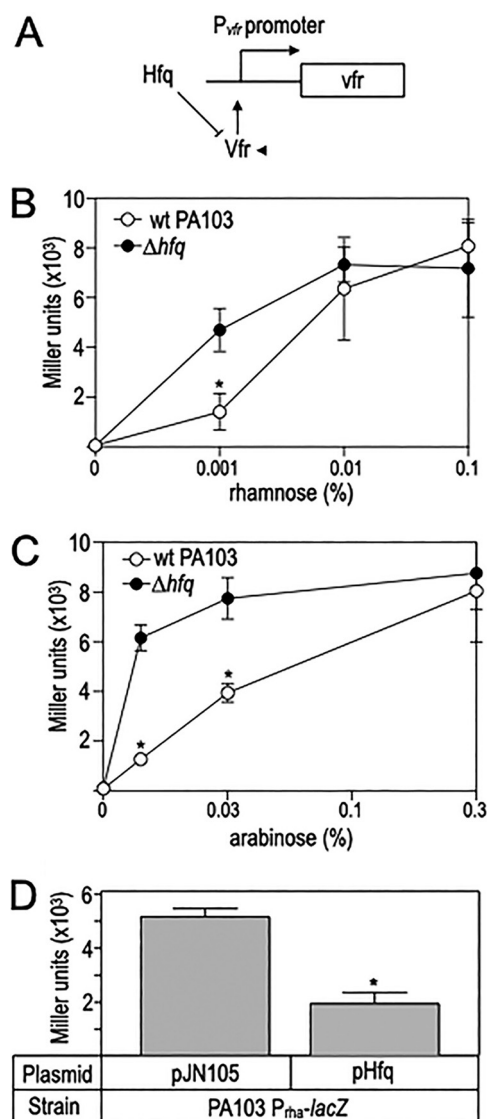
Since *vfr* transcription is autoregulated by Vfr, *vfr* expression is dependent upon cAMP (38). cAMP synthesis is controlled by the complex Pil-Chp chemosensory system and by



**FIG 4** Vfr directly binds to  $P_{Rha}$  and  $P_{BAD}$  promoter probes. (A) Wt PA103 and the  $\Delta vfr$  mutant carrying a chromosomally integrated  $P_{Rha}$ -*lacZ* transcriptional reporter lacking ( $- rhaT$ ) or including ( $+ rhaT$ ) the rhamnose transporter gene *rhaT* were cultured in LB with the indicated concentrations of rhamnose and assayed for  $\beta$ -galactosidase activity, reported in Miller units. The reported data are the averages from at least three independent experiments. (B and C) Radiolabeled  $P_{Rha}$  (B) and  $P_{BAD}$  (C) promoter probes were incubated alone (lane 1) or with the indicated concentrations of Vfr in the presence of the nonspecific competitor poly(dI-dC) and a second radiolabeled probe derived from *algD* that served as a negative control. Lane C is a control with the *algD* probe incubated with 200 nM Vfr. Reaction products were subjected to nondenaturing gel electrophoresis followed by phosphorimaging. The asterisk in panel B indicates the second promoter probe complex observed at the higher concentrations of Vfr tested. The phosphorimages are representative data from two independent experiments.

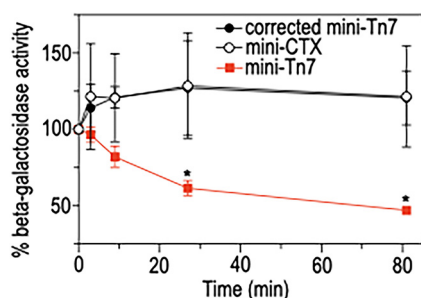
NadD2, both of which regulate the CyaB adenylate cyclase (39, 40). cAMP levels are also influenced by the second messenger cyclic di-GMP (c-di-GMP) (29). c-di-GMP homeostasis is controlled by diguanylate cyclases and phosphodiesterases, and there are  $\sim 40$  proteins with at least one of these activities in the *P. aeruginosa* genome. While our understanding of signals and mechanisms that control c-di-GMP homeostasis is incomplete, cAMP and cyclic di-GMP levels demonstrate an inverse relationship under some conditions (29, 41). Likewise, transcription and synthesis of Vfr is influenced by global regulatory systems, including the Rsm system, the AlgU sigma factor/MucA anti-sigma factor which control alginate biosynthesis, and the RNA chaperone Hfq and small noncoding RNAs (30–32). Finally, environmental signals, including NaCl, calcium, pH, osmolarity, and bicarbonate levels in the growth medium, can also impact cAMP-Vfr signaling (27, 42, 43). The numerous genetic background and environmental signals that directly or indirectly effect cAMP-Vfr signaling all have the potential to impact expression from the  $P_{Rha}$  and  $P_{BAD}$  promoters.

A previous study found that Vfr is also required for  $P_{Rha}$  promoter activity in *Pseudomonas putida* (37). The authors concluded that the Vfr requirement resulted from a



**FIG 5** Influence of Hfq on Vfr-dependent control of the  $P_{Rha}$  promoter. (A) Hfq inhibits Vfr synthesis. (B and C) PA103 wt and  $\Delta hfq$  strains carrying a chromosomally integrated  $P_{Rha}$ -lacZ (B) or  $P_{BAD}$ -lacZ (C) transcriptional reporter were cultured in LB with the indicated concentrations of rhamnose or arabinose, respectively. When the culture  $A_{600}$  reached 1.0, the cells were harvested and assayed for  $\beta$ -galactosidase activity, reported in Miller units. (D) PA103 carrying either empty vector control (pJN105) or an Hfq expression vector (pHfq) were cultured in LB with 0.2% arabinose and 0.005% rhamnose. Cells were assayed for  $\beta$ -galactosidase activity as described above. The reported data are the averages from at least three independent experiments. \*,  $P < 0.05$  (t test).

deficiency in rhamnose transport based on the findings that deletion of the Vfr (CRP)-binding site in the  $P_{Rha}$  promoter region had no effect on Vfr-dependent control and that providing a copy of the rhamnose transporter (*rhaT*) in *trans* restored rhamnose-dependent  $P_{Rha}$  promoter activity. Curiously, our findings in *P. aeruginosa* are the opposite. Direct binding of Vfr to the  $P_{Rha}$  and  $P_{BAD}$  promoter regions was demonstrated *in vitro* (Fig. 4B and C), and providing a copy of *rhaT* had no effect on  $P_{Rha}$  promoter activity in a  $\Delta vfr$  mutant (Fig. 4A). Our data are consistent with Vfr serving the same purpose as CRP does in *E. coli*, wherein Vfr/CRP directly participates in recruitment of RNAP at both the  $P_{Rha}$  and  $P_{BAD}$  promoters. The finding that inclusion of *rhaT* in a wt background potentiates  $P_{Rha}$  promoter activity in a dose-dependent manner suggests that rhamnose transport into *P. aeruginosa* is limiting. This is consistent with the lack of an identifiable rhamnose transporter in the genome and suggests that rhamnose uptake occurs through less efficient means. Limited rhamnose

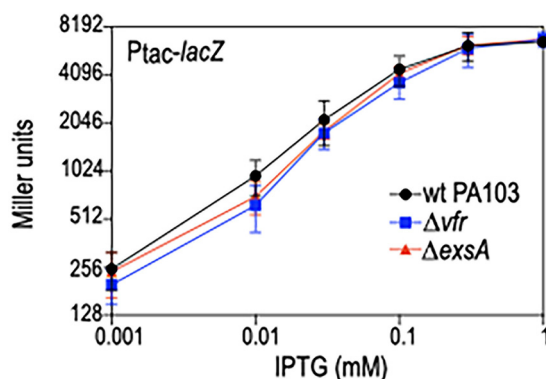


**FIG 6**  $\beta$ -Galactosidase expressed from pUC18-mini-Tn7T-Gm-LacZ10 is unstable. PA103 cells carrying the  $P_{Rha}$ - $lacZ$  reporter derived from pUC18-mini-Tn7T-Gm-LacZ10 (mini-Tn7), a  $P_{Rha}$ - $lacZ$  derivative with a corrected  $lacZ$  gene (corrected mini-Tn7), and a strain with a mini-CTX- $lacZ$  based reporter (mini-CTX) were harvested in a series of tubes containing Z buffer. Samples were assayed for  $\beta$ -galactosidase activity at the indicated times. The reported values represent the amount of  $\beta$ -galactosidase activity remaining at each time point relative to time zero. The reported data are the averages from least three independent experiments. \*,  $P < 0.05$  (ANOVA).

uptake raises several questions, including the identity of the transporter involved and whether transport and, by inference, expression from the  $P_{Rha}$  promoter are uniform within a cell population. The same questions also apply to the  $P_{BAD}$  promoter. Fortunately,  $P_{Tac}$  promoter activity is unaffected in a  $vfr$  mutant, and its use remains a reasonable approach to achieve regulated gene expression in *P. aeruginosa*. Nevertheless, any promoter subject to regulation, even  $P_{Tac}$ , should not be assumed to be equivalently expressed in different genetic backgrounds.

## MATERIALS AND METHODS

**Strain and plasmid construction.** The bacterial strains used in this study are listed in Table S1 in the supplemental material. Routine cloning was performed with *E. coli* DH5 $\alpha$  cultured in LB-Lennox medium with gentamicin (15  $\mu$ g/ml) as required. The pUC18 Tn7  $P_{Rha}$ - $lacZ$  reporter was constructed by PCR amplification of the  $P_{Rha}$  cassette from pSCPrhaB2 (44) using primers 196423236 and 196423237 (Table S2). Primer 196423237 introduces a unique PmlI restriction site at the  $P_{Rha}$  promoter transcription start site. The  $P_{Rha}$  cassette and a gBlock consisting of a  $lacZ$  adaptor were assembled into NsiI/BamHI-digested pUC18-mini-Tn7T-Gm-LacZ10 (Addgene plasmid 65026) using the Gibson assembly method, resulting in pUC18 Tn7  $P_{Rha}$ - $lacZ$ . The gene encoding the rhamnose transporter ( $rhaT$ ) driven by a  $P_{Tac}$  promoter was synthesized as a gBlock (pTAC  $rhaT$ ) (Table S2) and cloned into the NsiI site of pUC18 Tn7  $P_{Rha}$ - $lacZ$ , resulting in pUC18 Tn7  $P_{Rha}$ - $lacZ$  +  $rhaT$ . The pUC18 Tn7  $P_{BAD}$ - $lacZ$  reporter was constructed by PCR amplification of the  $P_{BAD}$  cassette from pJN105 using primers 288109006 and 288109007 (Table S2) and cloning into the NsiI and PmlI restriction sites of pUC18 Tn7  $P_{Rha}$ - $lacZ$ . The pUC18 Tn7  $P_{Tac}$ - $lacZ$  reporter was constructed by PCR amplification of the  $P_{Tac}$  cassette by using primers 293293895 and 293293896 (Table S2) and cloning into the NsiI and PmlI restriction sites of pUC18 Tn7  $P_{Rha}$ - $lacZ$ . Primers



**FIG 7**  $P_{Tac}$  promoter activity is Vfr independent. PA103 and a  $\Delta vfr$  mutant carrying a chromosomally integrated  $P_{Tac}$ - $lacZ$  transcriptional reporter were cultured in LB with the indicated concentrations of IPTG. When the culture  $A_{600}$  reached 1.0, the cells were harvested and assayed for  $\beta$ -galactosidase activity, reported in Miller units. The reported data are the averages from at least three independent experiments. There was no statistical difference (ANOVA) in  $P_{Tac}$ - $lacZ$  transcriptional reporter activity between PA103 and the  $\Delta vfr$  mutant.

and gBlocks were synthesized by Integrated DNA Technologies (Coralville, IA). pUC18 Tn7  $P_{\text{Rha}}\text{-lacZ}$  corrected was constructed by replacing the SacI-SapI restriction fragment containing the 3' end of *lacZ* with the corresponding SacI-SapI fragment from mini-CTX- $P_{\text{exsD}}\text{-lacZ}$  using the Gibson assembly method.

*P. aeruginosa* strains were maintained on Vogel-Bonner minimal (VBM) medium supplemented with gentamicin (80  $\mu\text{g/ml}$ ) or carbenicillin (300  $\mu\text{g/ml}$ ) as required. Tn7-integrating plasmids and helper plasmid pTNS2 (Addgene plasmid 64968) were introduced by electroporation. To remove the gentamicin resistance gene, pFLP2 (45) was introduced by electroporation and was resolved on yeast-tryptone medium supplemented with 10% sucrose.

**$\beta$ -Galactosidase assays.** *P. aeruginosa* was cultured overnight at 37°C in LB-Lennox medium containing gentamicin as required. Cells were diluted the next day to an absorbance ( $A_{600}$ ) of 0.1 in LB-Lennox medium with gentamicin as required and the concentrations of rhamnose, arabinose, and/or isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) indicated in the figure legends. Cells were incubated at 37°C. When the  $A_{600}$  reached 0.9 to 1.1, cells (100  $\mu\text{l}$ ) were mixed with 900  $\mu\text{l}$  of Z buffer (0.1 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  [pH 7.0], 10 mM KCl, 1 mM  $\text{MgSO}_4$ , 50 mM 2-mercaptoethanol), 20  $\mu\text{l}$  sodium dodecyl sulfate, and 20  $\mu\text{l}$  chloroform. Samples were lysed by vortexing at maximum speed for 10 s and then assayed for  $\beta$ -galactosidase activity using *ortho*-nitrophenyl- $\beta$ -galactoside (ONPG).  $\beta$ -Galactosidase activity was measured at  $A_{420}$  and calculated as Miller units (46). The reported values (Miller units) represent the average of at least three independent experiments. Statistical analyses were determined by one-way analysis of variance (ANOVA) with the Dunnett *post hoc* test or a *t* test using GraphPad Prism version 5.0c for Mac OS X (GraphPad Software, La Jolla, CA).

**Immunoblots.** Samples were prepared as previously described (47). Cell fractions were separated by 12% SDS-PAGE and analyzed by immunoblotting with primary antibodies to Vfr or ExsA in Tris-buffered saline with 5% skim milk and 0.1% Tween 20. Secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody) was used at a 1:8,000 dilution. Blots were imaged with an Azure Biosystems Sapphire imager using SuperSignal West Pico Plus substrate (Thermo Scientific).

**Electrophoretic mobility shift assays.**  $P_{\text{Rha}}$  (primers 292259572 and 292259573) and  $P_{\text{BAD}}$  (primers 292259570 and 292259571) promoter probes were PCR amplified and end labeled with 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}$ ] ATP as previously described (48). A 180-bp fragment derived from the coding region of *algD* served as the negative control, as previously reported (28). Specific and nonspecific promoter probes (0.05 nM) were incubated in DNA binding buffer (10 mM Tris-HCl pH [7.5], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol), 25 ng/ $\mu\text{l}$  poly(2'-deoxyinosinic-2'-deoxycytidylic acid), and 100  $\mu\text{g/ml}$  bovine serum albumin in a total volume of 19  $\mu\text{l}$  for 5 min at 25°C. Vfr was expressed and purified from *E. coli* by incubating a cell extract with cAMP-agarose, washing, and then eluting with an excess of cAMP as previously described (49). The resulting Vfr preparation is saturated with cAMP. Vfr was added in the indicated concentrations to a total volume of 20  $\mu\text{l}$  and incubated for an additional 15 min at 25°C. Reaction samples were then mixed with 1  $\mu\text{l}$  loading buffer (0.05% xylene cyanol, 50% glycerol) and analyzed by electrophoresis on 5% polyacrylamide glycine gels (10 mM Tris [pH 7.5], 380 mM glycine, 1 mM EDTA) at 4°C. Imaging was performed using an Azure Sapphire phosphorimager and software.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.04 MB.

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## REFERENCES

- Sheppard DE, Engleberg E. 1967. Further evidence for positive control of the L-arabinose system by gene *araC*. *J Mol Biol* 25:443–454. [https://doi.org/10.1016/0022-2836\(67\)90197-0](https://doi.org/10.1016/0022-2836(67)90197-0).
- Egan SM, Schleif RF. 1993. A regulatory cascade in the induction of *rhaBAD*. *J Mol Biol* 234:87–98. <https://doi.org/10.1006/jmbi.1993.1565>.
- Kolin A, Balasubramaniam V, Skredenske JM, Wickstrum JR, Egan SM. 2008. Differences in the mechanism of the allosteric L-rhamnose responses of the AraC/XylS family transcription activators RhaS and RhaR. *Mol Microbiol* 68:448–461. <https://doi.org/10.1111/j.1365-2958.2008.06164.x>.
- Schleif R. 2010. AraC protein, regulation of the L-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action. *FEMS Microbiol Rev* 34:779–796. <https://doi.org/10.1111/j.1574-6976.2010.00226.x>.
- Magasanik B. 1961. Catabolite repression. *Cold Spring Harbor Symp Quant Biol* 26:249–256. <https://doi.org/10.1101/sqb.1961.026.01.031>.
- Makman RS, Sutherland EW. 1965. Adenosine 3',5'-phosphate in *Escherichia coli*. *J Biol Chem* 240:1309–1314. [https://doi.org/10.1016/S0021-9258\(18\)97576-9](https://doi.org/10.1016/S0021-9258(18)97576-9).
- Zubay G, Schwartz D, Beckwith J. 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. *Proc Natl Acad Sci U S A* 66:104–110. <https://doi.org/10.1073/pnas.66.1.104>.
- Emmer M, DeCrombrughe B, Pastan I, Perlman R. 1970. Cyclic AMP receptor protein of *E. coli*: its role in the synthesis of inducible enzymes. *Proc Natl Acad Sci U S A* 66:480–487. <https://doi.org/10.1073/pnas.66.2.480>.
- Zheng D, Constantinidou C, Hobman JL, Minchin SD. 2004. Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. *Nucleic Acids Res* 32:5874–5893. <https://doi.org/10.1093/nar/gkh908>.
- Grainger DC, Hurd D, Harrison M, Holdstock J, Busby SJW. 2005. Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. *Proc Natl Acad Sci U S A* 102:17693–17698. <https://doi.org/10.1073/pnas.0506687102>.
- Shimada T, Fujita N, Yamamoto K, Ishihama A. 2011. Novel roles of cAMP receptor protein (CRP) in regulation of transport and metabolism of carbon sources. *PLoS One* 6:e20081. <https://doi.org/10.1371/journal.pone.0020081>.

12. Chen Y, Ebright YW, Ebright RH. 1994. Identification of the target of a transcription activator protein by protein-protein photocrosslinking. *Science* 265:90–92. <https://doi.org/10.1126/science.8016656>.
13. Rhodius VA, Busby SJW. 2000. Interactions between activating region 3 of the *Escherichia coli* cyclic AMP receptor protein and region 4 of the RNA polymerase  $\sigma$ 70 subunit: application of suppression genetics. *J Mol Biol* 299:311–324. <https://doi.org/10.1006/jmbi.2000.3737>.
14. Lee N, Wilcox G, Gielow W, Arnold J, Cleary P, Englesberg E. 1974. In vitro activation of the transcription of *araBAD* operon by *araC* activator. *Proc Natl Acad Sci U S A* 71:634–638. <https://doi.org/10.1073/pnas.71.3.634>.
15. Bass R, Heffernan L, Sweadner K, Englesberg E. 1976. The site for catabolite deactivation in the L-arabinose BAD operon in *Escherichia coli* B/r. *Arch Microbiol* 110:135–143. <https://doi.org/10.1007/BF00416978>.
16. Casadaban MJ. 1976. Regulation of the regulatory gene for the arabinose pathway, *araC*. *J Mol Biol* 104:557–566. [https://doi.org/10.1016/0022-2836\(76\)90120-0](https://doi.org/10.1016/0022-2836(76)90120-0).
17. Miyada CG, Stoltzfus L, Wilcox G. 1984. Regulation of the *araC* of *Escherichia coli*: catabolite repression, autoregulation, and effect on *araBAD* expression. *Proc Natl Acad Sci U S A* 81:4120–4124. <https://doi.org/10.1073/pnas.81.13.4120>.
18. Lichenstein HS, Hamilton EP, Lee N. 1987. Repression and catabolite gene activation in the *araBAD* operon. *J Bacteriol* 169:811–822. <https://doi.org/10.1128/jb.169.2.811-822.1987>.
19. Stoltzfus L, Wilcox G. 1989. Effect of mutations in the cyclic AMP receptor protein-binding site on *araBAD* and *araC* expression. *J Bacteriol* 171:1178–1184. <https://doi.org/10.1128/jb.171.2.1178-1184.1989>.
20. Holcroft CC, Egan SM. 2000. Interdependence of activation at *rhaSR* by cyclic AMP receptor protein, the RNA polymerase alpha subunit C-terminal domain, and RhaR. *J Bacteriol* 182:6774–6782. <https://doi.org/10.1128/jb.182.23.6774-6782.2000>.
21. Wickstrum JR, Santangelo TJ, Egan SM. 2005. Cyclic AMP receptor protein and RhaR synergistically activate transcription from the L-rhamnose-responsive *rhaSR* promoter in *Escherichia coli*. *J Bacteriol* 187:6708–6718. <https://doi.org/10.1128/JB.187.19.6708-6718.2005>.
22. Suh SJ, Runyen-Janecky LJ, Maleniak TC, Hager P, MacGregor CH, Zielinski-Mozny NA, Phibbs PV, West SEH. 2002. Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. *Microbiology (Reading)* 148:1561–1569. <https://doi.org/10.1099/00221287-148-5-1561>.
23. West SEH, Sample AK, Runyen-Janecky LJ. 1994. The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. *J Bacteriol* 176:7532–7542. <https://doi.org/10.1128/JB.176.24.7532-7542.1994>.
24. Davinic M, Carty NL, Colmer-Hamood JA, San Francisco M, Hamood AN. 2009. Role of *vfr* in regulating exotoxin A production by *Pseudomonas aeruginosa*. *Microbiology (Reading)* 155:2265–2273. <https://doi.org/10.1099/mic.0.028373-0>.
25. Albus AM, Pesci EC, Runyen-Janecky LJ, West SE, Iglewski BH. 1997. Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179:3928–3935. <https://doi.org/10.1128/jb.179.12.3928-3935.1997>.
26. Beatson SA, Whitchurch CB, Sargent JL, Levesque RC, Mattick JS. 2002. Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J Bacteriol* 184:3605–3613. <https://doi.org/10.1128/jb.184.13.3605-3613.2002>.
27. Wolfgang MC, Lee VT, Gilmore ME, Lory S. 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* 4:253–263. [https://doi.org/10.1016/S1534-5807\(03\)00019-4](https://doi.org/10.1016/S1534-5807(03)00019-4).
28. Marsden AE, Intile PJ, Schulmeyer KH, Simmons-Patterson ER, Urbanowski ML, Wolfgang MC, Yahr L. 2016. Vfr directly activates *exsA* transcription to regulate expression of the *Pseudomonas aeruginosa* type III secretion system. *J Bacteriol* 198:1442–1450. <https://doi.org/10.1128/JB.00049-16>.
29. Almlad H, Harrison JJ, Rybtke M, Groizeleau J, Givskov M, Parsek MR, Tolker-Nielsen T. 2015. The cyclic AMP-Vfr signaling pathway in *Pseudomonas aeruginosa* is inhibited by cyclic di-GMP. *J Bacteriol* 197:2190–2200. <https://doi.org/10.1128/JB.00193-15>.
30. Irie Y, La Mensa A, Murina V, Hauriuk V, Tenson T, Shingler V. 2020. Hfq-assisted RsmA regulation is central to *Pseudomonas aeruginosa* biofilm polysaccharide PEL expression. *Front Microbiol* 11:2720. <https://doi.org/10.3389/fmicb.2020.482585>.
31. Jones AK, Fulcher NB, Balzer GJ, Urbanowski ML, Pritchett CL, Schurr MJ, Yahr TL, Wolfgang MC. 2010. Activation of the *Pseudomonas aeruginosa* AlG<sub>U</sub> regulon through *mucA* mutation inhibits cyclic AMP/Vfr signaling. *J Bacteriol* 192:5709–5717. <https://doi.org/10.1128/JB.00526-10>.
32. Janssen KH, Corley JM, Djapgne L, Cribbs JT, Voelker D, Slusher Z, Nordell R, Regulski EE, Kazmierczak BI, McMackin EW, Yahr TL. 2020. Hfq and sRNA 179 inhibit expression of the *Pseudomonas aeruginosa* cAMP-Vfr and type III secretion regulons. *mBio* 11:e00363-20. <https://doi.org/10.1128/mBio.00363-20>.
33. de Boer HA, Comstock LJ, Vasser M. 1983. The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. *Proc Natl Acad Sci U S A* 80:21–25. <https://doi.org/10.1073/pnas.80.1.21>.
34. Silverstone AE, Arditti RR, Magasanik B. 1970. Catabolite-insensitive revertants of *lac* promoter mutants. *Proc Natl Acad Sci U S A* 66:773–779. <https://doi.org/10.1073/pnas.66.3.773>.
35. Newman JR, Fuqua C. 1999. Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli* *araBAD* promoter and the *araC* regulator. *Gene* 227:197–203. [https://doi.org/10.1016/S0378-1119\(98\)00601-5](https://doi.org/10.1016/S0378-1119(98)00601-5).
36. McCaw ML, Lykken GL, Singh PK, Yahr TL. 2002. ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol Microbiol* 46:1123–1133. <https://doi.org/10.1046/j.1365-2958.2002.03228.x>.
37. Jeske M, Altenbuchner J. 2010. The *Escherichia coli* rhamnose promoter *rhaPBAD* is in *Pseudomonas putida* KT2440 independent of Crp-cAMP activation. *Appl Microbiol Biotechnol* 85:1923–1933. <https://doi.org/10.1007/s00253-009-2245-8>.
38. Fuchs EL, Brutinel ED, Jones AK, Fulcher NB, Urbanowski ML, Yahr TL, Wolfgang MC. 2010. The *Pseudomonas aeruginosa* Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. *J Bacteriol* 192:3553–3564. <https://doi.org/10.1128/JB.00363-10>.
39. Fulcher NB, Holliday PM, Klem E, Cann MJ, Wolfgang MC. 2010. The *Pseudomonas aeruginosa* Chp chemosensory system regulates intracellular cAMP levels by modulating adenylate cyclase activity. *Mol Microbiol* 76:889–904. <https://doi.org/10.1111/j.1365-2958.2010.07135.x>.
40. Jin Y, Zhang M, Zhu F, Peng Q, Weng Y, Zhao Q, Liu C, Bai F, Cheng Z, Jin S, Wu W. 2019. NrtR regulates the type III secretion system through cAMP/Vfr pathway in *Pseudomonas aeruginosa*. *Front Microbiol* 10:85. <https://doi.org/10.3389/fmicb.2019.00085>.
41. Almlad H, Rybtke M, Hendiani S, Andersen JB, Givskov M, Tolker-Nielsen T. 2019. High levels of cAMP inhibit *Pseudomonas aeruginosa* biofilm formation through reduction of the c-di-GMP content. *Microbiology (Reading)* 165:324–333. <https://doi.org/10.1099/mic.0.000772>.
42. Dobay O, Laub K, Stercz B, Kéri A, Balázs B, Tóthpál A, Kardos S, Jaikumpun P, Ruksakiet K, Quinton PM, Zsembely Á. 2018. Bicarbonate inhibits bacterial growth and biofilm formation of prevalent cystic fibrosis pathogens. *Front Microbiol* 9:2245. <https://doi.org/10.3389/fmicb.2018.02245>.
43. Rietsch A, Mekalanos JJ. 2006. Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* 59:807–820. <https://doi.org/10.1111/j.1365-2958.2005.04990.x>.
44. Cardona ST, Valvano MA. 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. *Plasmid* 54:219–228. <https://doi.org/10.1016/j.plasmid.2005.03.004>.
45. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86. [https://doi.org/10.1016/S0378-1119\(98\)00130-9](https://doi.org/10.1016/S0378-1119(98)00130-9).
46. Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
47. McMackin EAW, Marsden AE, Yahr TL. 2019. H-NS family members MvaT and MvaU regulate the *Pseudomonas aeruginosa* type III secretion system. *J Bacteriol* 201:e00054-19. <https://doi.org/10.1128/JB.00054-19>.
48. Brutinel ED, Vakulskas CA, Brady KM, Yahr TL. 2008. Characterization of ExsA and of ExsA-dependent promoters required for expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* 68:657–671. <https://doi.org/10.1111/j.1365-2958.2008.06179.x>.
49. Fuchs EL, Brutinel ED, Klem ER, Fehr AR, Yahr TL, Wolfgang MC. 2010. In vitro and in vivo characterization of the *Pseudomonas aeruginosa* cyclic AMP (cAMP) phosphodiesterase CpdA, required for cAMP homeostasis and virulence factor regulation. *J Bacteriol* 192:2779–2790. <https://doi.org/10.1128/JB.00168-10>.